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TN 38103 (US). SCHUETZ, John [US/US]; 1442 Carr Avenue, Memphis, TN 38105 (US).

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(74) Agent: JACKSON, David, A.; Klauber & Jackson, 411 Hackensack Avenue, Hackensack, NJ 07601 (US).

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(71) Applicant (*for all designated States except US*): ST. JUDE CHILDREN'S RESEARCH HOSPITAL [US/US]; 332 North Lauderdale Street, Memphis, TN 38105-2794 (US).

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(72) Inventors; and

(75) Inventors/Applicants (*for US only*): SORRENTINO, Brian [US/US]; 6590 Quail Point Circle North, Memphis,

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(54) Title: METHOD OF IDENTIFYING AND/OR ISOLATING STEM CELLS AND PROGNOSING RESPONSIVENESS TO LEUKEMIA TREATMENT

(57) Abstract: The present invention includes methods of identifying and/or isolating stem cells based on expression of BCRP. The present invention also describes methods of obtaining and/or using cell populations enriched for stem cells. In addition, methods are provided for diagnosing and/or prognosing leukemia, particularly human acute myelogenous leukemia (AML), through assaying for BCRP expression in leukemic cells.

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**METHOD OF IDENTIFYING AND/OR ISOLATING STEM CELLS AND
PROGNOSING RESPONSIVENESS TO LEUKEMIA TREATMENT**

5

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15

FIELD OF THE INVENTION

The present invention provides a method of identifying and/or isolating stem cells. The present invention also provides methods of using cell populations enriched for stem cells. In addition, the present invention provides methods for diagnosing and/or
20 prognosing human acute myelogenous leukemia (AML).

BACKGROUND OF THE INVENTION

All of the cells and cell types of an individual adult mammal are derived from a single
25 cell, the zygote. However, as cells mature and differentiate they lose their ability to be converted into a different cell type. Thus, most adult cells are fully differentiated and normally cannot be converted into another cell type. One particular exception is the adult stem cell. Adult stem cells retain the ability to differentiate into other cell types, though this differentiation is generally limited to forming cells of a single tissue type.
30 For example, hematopoietic stem cells (HSCs) are capable of differentiating into any cell type of the blood and immune system, whereas brain stem cells can differentiate into the different cell types of the brain. In recent years, therapies for treating degenerative diseases and/or cancer (such as leukemia) have been designed which employ stem cells. However, heretofore, isolating stem cells from the human donors
35 has proved to be extremely difficult since stem cells are relatively rare.

Hematopoietic stem cells are functionally defined based on their capacity for self-renewal divisions, which leads to the continuous generation of new HSCs over the lifetime of an animal, and by their potential for pluripotent hematopoietic differentiation. There are three possible general outcomes for the resulting daughter cells when a hematopoietic stem cell divides: (i) differentiation, (ii) self-renewal, or (iii) apoptosis. Despite the extensive study of HSCs, due to its relevance to bone marrow transplantation, gene therapy, and basic hematopoiesis, the mechanisms controlling these three tightly regulated outcomes are poorly understood.

Purification strategies for HSCs have been developed for both mouse [Spangrude *et al.*, *Science* 241:58-62 (1988); (published erratum appears in *Science* 244(4908):1030 (1989)); Uchida *et al.*, *J.Exp.Med.* 175:175-184 (1992)] and humans HSCs [Zanjani *et al.*, *J.Clin.Invest.* 93:1051-1055 (1994), *see comments*; Larochelle *et al.*, *Nat.Med.* 2:1329-1337 (1996); Civin *et al.*, *Blood* 88:4102-4109 (1996)]. Most of these strategies use antibodies directed against various cell surface antigens and multiparameter cell sorting to isolate phenotypically defined cell populations. This approach has allowed isolation of murine stem cell populations of sufficiently high purity to allow reconstitution of irradiated recipients with less than 10 cells [Morrison *et al.*, *Proc.Natl.Acad.Sci.USA* 92:10302-10306 (1995); Osawa *et al.*, *Science* 273:242-245 (1996)], while considerably greater numbers of sorted human cells have been required to reconstitute xenogeneic recipients [Larochelle *et al.*, *Nat.Med.* 2:1329-1337 (1996); Zanjani *et al.*, *Exp.Hematol.* 26:353-360 (1998), *see comments*]. The human MDR1 gene and its murine homologs were originally identified based on the ability of their expressed products, collectively referred to as P-glycoproteins (P-gps), to extrude a wide variety of cytotoxic drugs from the cell interior [Gros *et al.*, *Cell*, 47:371-380 (1986) and Chen *et al.*, *Cell*, 47:381-389 (1986)]. It is now known that the MDR1 gene belongs to a superfamily of transport proteins that contain a conserved ATP-binding cassette (ABC) which is necessary for pump function [Allikmets *et al.*, *Hum. Mol. Genet.* 5:1649-1655 (1996)]. Numerous studies have clearly shown that P-gp expression plays an important role in the resistance of human tumor cells to cancer chemotherapy [Pastan and Gottesman, *Annu. Rev. Med.*, 42:277-

286 (1991)]. Considering that P-gps are also expressed in a wide variety of normal tissues, more recent studies have examined the normal physiologic functions of MDR1-like genes. Murine gene disruption experiments have demonstrated that expression of various P-gps is necessary for biliary excretion [Smit *et al.*, *Cell*,
5 75:451-462 (1993)], maintenance of the blood-brain barrier [Schinkel *et al.*, *Cell*, 77:491-502 (1994)], and elimination of drugs [Schinkel *et al.*, *Proc.Natl.Acad.Sci. USA*, 94:4028-4033 (1997)]. P-gps can also mediate more general cellular functions including the translocation of lipids across the cell membrane [van Helvoort *et al.*, *Cell*, 87:507-517 (1996)] and modulation of specific apoptosis pathways [Johnstone *et al.*, *Blood*, 93:1075-1085 (1999) and Smyth *et al.*, *Proc.Natl.Acad.Sci.USA*, 95:7024-
10 7029 (1998)].

P-gp is expressed in a variety of hematopoietic cell types [Drach *et al.*, *Blood*, 80:2729-2734 (1992)], including human CD34⁺ stem cells [Chaudhary and Roninson,
15 *Cell*, 66:85-94 (1991)] and murine c-kit⁺ stem cells [Sorrentino *et al.*, *Blood*, 86:491-501 (1995)]. Several lines of evidence suggest that P-gp expression is functionally conserved in hematopoietic stem cells.

Another ATP transport protein that contains a conserved ATP-binding cassette is the
20 gene product of the *Bcrp1/Mxr/Abcp/ABCG2* gene (referred to herein as BCRP when obtained from any mammalian source, but as mBCRP and huBCRP when the specific mouse or human gene or gene product(s) are being particularly referenced). The huBCRP cDNA was originally cloned from several different human tumor cell lines that were resistant to multiple drugs including doxorubicin, topotecan, and
25 mitoxantrone [Doyle *et al.*, *Proc.Natl.Acad.Sci.USA* 95:15665-15670 (1998):(published erratum appears in *Proc Natl Acad Sci U S A*; 96(5):2569 (1999)); Maliepaard *et al.*, *Cancer Res.* 59:4559-4563 (1999); Miyake *et al.*, *Cancer Res.* 59:8-13 (1999)]. A highly related mouse homologue (*mBcrp1*) was cloned from fibroblasts selected for multidrug resistance [Allen *et al.*, *Cancer Res.* 59:4237-4241
30 (1999)]. In contrast to the structure of the MDR1 gene, which consists of two duplicated halves, the predicted structure of BCRP is that of a "half transporter", with a single ATP binding cassette and transmembrane region. The expression pattern of

human BCRP (huBCRP) is highly restricted in normal human tissues, with the highest levels of mRNA detected in the placenta, and much lower levels detected in adult organs [Doyle *et al.*, *Proc.Natl.Acad.Sci.USA* 95:15665-15670 (1998);(published erratum appears in *Proc.Natl.Acad.SciUSA* 96(5):2569 (1999)); Allikmets *et al.*,
5 *CancerRes.* 58:5337-5339 (1998)].

Hematopoietic stem cells can be identified based on their ability to efflux fluorescent dyes that are substrates for P-gp, such as Rhodamine (Rho) 123 [Spangrude and Johnson, *Proc.Natl.Acad.Sci. SA*, 87:7433-7437 (1990); Fleming *et al.*, *J. Cell Biol.*,
10 122:897-902 (1993); Orlic *et al.*, *Blood*, 82:762-770 (1993); and Zijlmans *et al.*, *Proc.Natl.Acad.Sci.USA*, 92:8901-8905 (1995)] and Hoechst 33342 [McAlister *et al.*, *Blood*, 75:1240-1246 (1990); Wolf *et al.*, *Exp. Hematol.*, 21:614-622 (1993); and Leemhuis *et al.*, *Exp. Hematol.*, 24:1215-1224 (1996)]. One particular approach for purifying stem cells is based on Hoechst dye-staining of bone marrow cells to identify
15 a minor fraction of side population (SP) cells that are highly enriched for repopulating activity [Goodell *et al.*, *J. Exp. Med.*, 183:1797-1806 (1996)]. This SP phenotype identifies a primitive subset of stem cells present in multiple mammalian species [Goodell *et al.*, *Nat. Med.*, 3:1337-1345 (1997)], and based on verapamil inhibition studies, may be due to expression of P-gp or another ABC transporter [Goodell *et al.*,
20 *J. Exp. Med.*, 183:1797-1806 (1996)].

Despite a recent report demonstrating that sorting for expression of the vascular endothelial growth factor receptor can enrich human stem cells to near purity [Ziegler *et al.*, *Science* 285:1553-1558 (1999)], there still remains a general need for better and
25 more specific markers of human HSCs. In addition, there is a great need for new methodologies of isolating stem cells.

The citation of any reference herein should not be deemed as an admission that such reference is available as prior art to the instant invention.

SUMMARY OF THE INVENTION

The present invention provides methods of identifying and/or purifying stem cells by detecting and/or selecting for cells that express a specific transmembrane efflux pump.

- 5 More particularly the present invention discloses that BCRP expression is a more specific marker for stem cells than all currently available markers. Such stem cells include hematopoietic stem cells, and side population (SP) stem cells from other organs. The present invention therefore provides methods for isolating primitive stem cells based on the detection of BCRP expression, which as disclosed herein, is a
- 10 functional determinant for stem cells from a variety of tissues.

- The present invention further provides methods of identifying cells that express BCRP. One such embodiment comprises obtaining a cell sample which contains (or is suspected to contain) stem cells and detecting the expression of BCRP by a cell in
- 15 the cell sample. A cell is identified as a stem cell if BCRP is expressed by the cell. The detection of the expression of BCRP can be performed *via* its specific pumping activity, *i.e.*, it can remove Hoechst 33342 dye but not Rhodamine 123 dye (*see* Example 1 below). Preferably detection of the expression of BCRP is performed with an anti-BCRP antibody which binds to BCRP (more preferably the extracellular
- 20 portion of BCRP). Stem cells can then be identified due to their binding to the anti-BCRP antibody. In one such embodiment the antibody is a polyclonal antibody. In another embodiment the antibody is a monoclonal antibody. Alternatively, the detection of the expression of BCRP is performed *via* PCR employing a PCR probe derived for a nucleic acid sequence that expresses BCRP (*see* Example 1 below).

- 25 In an alternative embodiment, the present invention provides a method for identifying a stem cell that further comprises detecting the expression of one or more additional stem cell markers, *i.e.*, a protein that is expressed by stem cells. A cell that expresses BCRP and expresses one or more of such stem cell markers is then confirmed as a
- 30 stem cell. In one such embodiment the stem cell marker is EM10. In another embodiment the stem cell marker is CD34. In a preferred embodiment of this type the

stem cell is also CD38⁻ (*i.e.*, does not express CD38). In still another embodiment the stem cell marker is Thy-1. In yet another embodiment the stem cell marker is P-gp. In still another embodiment the stem cell marker is c-kit. In yet another embodiment the stem cell marker is Ac133.

5

The present invention also provides methods of further enriching the stem cell population in a mixture of cells that have previously been enriched by selecting for an alternative stem cell marker. In a particular embodiment of this type, the cells have been previously selected for the expression of the CD34 marker. Such an enriched
10 stem cell population can then be further enriched by selecting for cells that also express BCRP. Any of the methods taught by the present invention for identifying/selecting cells that express BCRP can be used .

Alternatively, or in conjunction with detecting the expression of BCRP, the methods
15 for identifying a stem cell of the present invention can further comprise detecting the expression of one or more lineage specific markers. A cell that expresses BCRP (and preferably one or more additional stem cell markers) but does not express the lineage specific marker(s) is then identified as a stem cell.

20 In a particular embodiment the stem cell is a hematopoietic stem cell. In one such embodiment the lineage specific marker whose absence is used to confirm stem cell identification is CD14. In another embodiment the lineage specific marker is CD15. In yet another embodiment the lineage specific marker is CD38. In still another embodiment the lineage specific marker is HLA-DR. In yet another embodiment the
25 lineage specific marker is CD71. In still another embodiment the lineage specific marker is CD33. In yet another embodiment the lineage specific marker is CD2. In still another embodiment the lineage specific marker is CD16. In yet another embodiment the lineage specific marker is CD19. In still another embodiment the lineage specific
30 marker is glycophorin A. In still another embodiment the lineage specific marker is CD3. In yet another embodiment the lineage specific marker is CD4. In still another

embodiment the lineage specific marker is CD8. In yet another embodiment the lineage specific marker is CD56.

5 The present invention therefore also provides methods of isolating stem cells. One such embodiment comprises a method of isolating a cell that expresses BCRP that comprises obtaining a cell sample which contains (or is suspected to contain) a cell that expresses BCRP and detecting the expression of BCRP by a cell in the cell sample. After being detected the cell that expresses BCRP is isolated. As indicated above, the absence and/or presence of lineage specific markers and stem cell markers
10 respectively, can also be employed in the detection of the stem cells.

Cell samples can be obtained from any animal, but preferably a mammal and more preferably a human. Preferably, these samples already have a cell population that has been previously enriched in stem cells.

15

One particular embodiment comprises obtaining a cell sample which contains (or is suspected to contain) stem cells and contacting them with an antibody that binds to BCRP (preferably an extracellular portion BCRP). Cells that bind to the antibody are then isolated. These isolated cells are identified as isolated stem cells due to their
20 binding to the anti-BCRP antibody. In a preferred embodiment of the present invention the isolation of the stem cells is performed by flow cytometry. In one particular embodiment, the antibody has a fluorescent label and the isolation of the stem cells is performed by fluorescent-activated cell sorting (FACS).

25 In another embodiment, the anti-BCRP antibody is placed on a solid support. The solid support can then be contacted/incubated with a sample of cells, such that the cells can associate with the solid support by binding to the anti-BCRP antibody. The solid support is then washed to remove cells that bind non-specifically. The remaining cells are eluted from the solid support (by an excess of free antibody, for
30 example). Based on their ability to bind anti-BCRP antibody with specificity, the eluted cells are identified as isolated stem cells.

In a particular embodiment, the solid support is an immunomagnetic bead (*e.g.*, MILTENYI MINIMACS™, DYNABEADS™). The anti-BCRP antibody is placed on the immunomagnetic beads which are then contacted/incubated with a sample of cells, as indicated above, such that the cells can associate with the beads by binding to the anti-BCRP antibody. Preferably after an appropriate incubation period, the immunomagnetic beads can then be separated from the sample of cells with a magnet. The immunomagnetic beads are then washed to remove cells that bind non-specifically. The remaining cells are eluted from the immunomagnetic beads as indicated above. Again, based on their ability to bind anti-BCRP antibody, the isolated cells are identified as stem cells.

In one embodiment the BCRP is a huBCRP gene product which is encoded by the nucleotide sequence of SEQ ID NO:9 and has the amino acid sequence of SEQ ID NO:10. In another embodiment the BCRP is a huBCRP gene product which is encoded by the nucleotide sequence of SEQ ID NO:26 and has the amino acid sequence of SEQ ID NO:27. In yet another embodiment the BCRP is a murine BCRP (mBCRP) which is encoded by the nucleotide sequence of SEQ ID NO:13 and has the amino acid sequence of SEQ ID NO:14. In still another embodiment the BCRP is a mBCRP which is encoded by a nucleotide sequence comprising SEQ ID NO:11 and has an amino acid sequence comprising SEQ ID NO:12.

Furthermore, any step in any method for isolating and/or identifying stem cells can be repeated to enhance the isolation/identification process. In addition, individual methods can also be combined in order to enhance the isolation/identification processes.

The purified/isolated stem cells obtained from the methodology of the present invention are also part of the present invention. In addition, cell populations enriched for stem cells obtained by a method of the present invention are also provided. One such enriched population is obtained from a mixed population of cells that comprises both stem cells and cells that are not stem cells. The cells of the mixed population of cells are then separated into two individual groups of cells based on whether or not

they express BCRP. The group of cells selected for expressing BCRP are a cell population enriched for stem cells. As indicated above, the selection process can be repeated one or more times to further enrich the population of cells for stem cells.

- 5 The identification and isolation of stem cells *via* the methods of the present invention extend beyond hematopoietic stem cells and comprises all stem cells, including but not limited to muscle stem cells, liver stem cells, gastrointestinal stem cells, brain stem cells, and embryonic stem cells. The present invention also provides methods of using these isolated stem cells including the use of muscle stem cells in the treatment
- 10 of diseases such as muscular dystrophy, degenerative liver disorder, myocardial infarction, Parkinson's disease, degenerative disorders of the brain, and for tissue regeneration/replacement. In addition, the hematopoietic stem cells can be used in bone marrow transplants (*e.g.*, for treatment of leukemia) as well as for *ex vivo* gene therapy for treatment of blood diseases such as sickle cell anemia and thalassemia.

15

- The present invention also provides the antibodies and portions thereof that recognize an extracellular portion of a BCRP in its natural conformation. In a preferred embodiment an antibody of the present invention recognizes BCRP on the surface of a viable stem cell. In a particular embodiment the antibody is a polyclonal antibody. In
- 20 another embodiment the antibody is a monoclonal antibody. In another embodiment the antibody is a chimeric antibody. In a preferred embodiment of this type the chimeric antibody is a humanized antibody.

- The present invention also provides a method of prognosing human leukemia, particularly acute myelogenous leukemia (AML), through determination of BCRP
- 25 expression in leukemic cells, *e.g.*, blast cells from individuals having or suspected of having AML. The effectiveness of chemotherapeutic agents which can be effluxed from the cell by BCRP is predictably decreased by the presence of BCRP on leukemic cells. Determination of the presence and level of BCRP expression in the leukemic
- 30 cells of an individual can therefore help the medical practitioner develop a therapeutic regimen which takes into account the predicted efficacy of such agents and allows

proper consideration of alternative therapies or agents which are not susceptible to the counter-effects of BCRP where BCRP expression is present.

Accordingly, it is a principal object of the present invention to provide a method of
5 obtaining purified stem cells.

It is a further object of the present invention to provide the purified stem cells.

It is a further object of the present invention to provide methods of using the purified
10 stem cells in the treatment of diseases in which one or more specific cell types are being adversely depleted and/or become dysfunctional.

It is a further object of the present invention to provide methods of using the purified stem cells for gene therapy.
15

It is a further object of the present invention to provide methods of identifying stem cells *in vitro*, *in situ*, and *in vivo* among mixed populations of cells and/or tissues.

It is a further object of the present invention to use BCRP as a marker for prognosing
20 the progression of AML.

These and other aspects of the present invention will be better appreciated by reference to the following drawings and Detailed Description.

25

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 is a schematic representation of a method of producing anti-BCRP monoclonal antibodies that can be used to isolate living stem cells by cell sorting.

30

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides the use of a specific ATP transporter, BCRP to identify and/or isolate a mammalian stem cell. Indeed, as shown herein, the BCRP gene is expressed at relatively high levels both in primitive CD34-murine HSCs and in SP cells from the bone marrow. In contrast, the expression of other known ABC transporters in the highly enriched CD34-stem cell population is low to absent. A retroviral vector expressing the huBCRP cDNA has been constructed as described below to study the functional properties of huBCRP. Fibroblasts expressing this vector gain the capacity to efflux Hoechst dye, a prerequisite property for establishment of the SP phenotype. Furthermore, when primary bone marrow cells are transduced with the BCRP vector, there is a large expansion of SP cells over time in culture. SP cells from the muscle also express BCRP at high levels. Indeed, BCRP mRNA expression is highly restricted in normal tissues. Furthermore, as disclosed herein, BCRP expression is relatively restricted to the hematopoietic stem cell compartment in mice. In accordance with these data BCRP expression is contemplated to be a universal marker for stem cells from various organs, and may well be the critical molecule for conferring the dye efflux phenotype to stem cells. Therefore, the present invention provides a method of identifying stem cells by their expression of BCRP. Such identification can then be used to isolate the stem cells.

Given the capacity of BCRP to confer resistance to anthracycline drugs [Miyake *et al.*, *Cancer Res.* 59:8-13 (1999)], BCRP expression may directly confer resistance to AML induction chemotherapy. Indeed, BCRP expression in AML blasts appears to be associated with a drug resistant phenotype and thereby predict a poor prognosis. Therefore, the present invention also provides a method of prognostigating pediatric patients with AML, *e.g.*, by examining/monitoring blast cells from such pediatric patients.

As demonstrated in the Examples below, stem cells from the bone marrow, skeletal muscle, and other tissues can be identified by the "side population" (SP) phenotype. While heretofore it had been assumed that the expression of ABC transporters is

responsible for this phenotype, the specific molecules involved had not been defined. Herein, it is demonstrated that expression of the *Bcrp1* (mBCRP)/ABCG2 (huBCRP) gene is a conserved feature of stem cells from diverse tissue sources. Indeed, mBCRP mRNA is expressed at high levels in primitive murine hematopoietic stem cells, and is sharply downregulated with stem cell differentiation. Enforced expression of the huBCRP cDNA directly conferred the SP phenotype to bone marrow cells, and causes a block in differentiation using both *in vitro* and transplantation-based assays. These results identify BCRP gene expression as a novel stem cell marker, and that its observed expression reflects a functional role in inhibiting stem cell differentiation. Therefore, the present invention provides methods of identifying stem cells through assays that can detect the expression of the BCRP.

Thus the studies disclosed herein demonstrate that expression of the BCRP transporter is highly conserved in primitive stem cells from a variety of sources. Expression was noted in SP cells from murine bone marrow, skeletal muscle, cultured ES cells, and in Rhesus monkey bone marrow. Within the lineage negative compartment of murine bone marrow cells, BCRP expression was relatively restricted to primitive CD34-stem cells, and expression was sharply downregulated with differentiation. The link between BCRP expression and SP cells was further strengthened by the observation that enforced expression in bone marrow cells caused a significant expansion of cells bearing the SP phenotype, both *in vitro* and *in vivo*. Altogether, these findings support the idea that BCRP expression is a widely conserved and specific marker for stem cells, and indicate that this transporter plays a role in stem cell function and/or maintenance of stem cell phenotype. Cell sorting for BCRP expression using appropriate antibodies provides a new strategy for stem cell purification applicable to cells from different organ sources. BCRP expression is further contemplated as a marker for the recently described trans-differentiating stem cells found in murine bone marrow [Lagasse, E. *et al.*, *Nat. Med.* 6:1229-1234 (2000)] and skeletal muscle [Jackson, K.A *et al.*, *Proc. Natl. Acad. Sci. U. S. A* 96:14482-14486 (1999); Gussoni, E. *et al.*, *Nature* 401: 390-394 (1999)].

Therefore, if appearing herein, the following terms shall have the definitions set out below.

- 5 A "hematopoietic stem cell" is a pluripotent cell that is able to either replicate itself with self-renewal divisions or to differentiate along a number of pathways and thereby generate erythrocytes, granulocytes, monocytes, mast cells, lymphocytes, and megakaryocytes. These stem cells occur with a frequency of about 1 stem cell per 10^4 bone marrow cells.
- 10 A "heterologous gene" as used herein is a gene that is introduced into a stem cell (*e.g.*, a hematopoietic stem cell) through a molecular biological manipulation. As defined herein, this molecular biological manipulation is made such that the heterologous gene is inserted into the stem cell. The heterologous gene need not be expressed in the stem cell as long as it is expressed in the progeny of the stem cell. The coding
15 sequence of the heterologous gene is operatively linked to an expression control sequence. Generally a heterologous gene is first placed into a vector. The heterologous gene is not necessarily naturally contained by the vector, though a heterologous gene can encode a protein that is native to the stem cell. For example, the heterologous gene can encode a functional protein and be used in *ex vivo* gene
20 therapy to replace the corresponding defective gene in a stem cell, *e.g.*, an hematopoietic stem cell. The heterologous gene will usually be flanked by DNA that does not flank the genomic DNA in the genome of the source organism. Alternatively, the heterologous gene may not be naturally found in the stem cell, such as the gene for human MDR1 introduced into a murine hematopoietic stem cell.
- 25 A cell has been "transduced" by a heterologous gene such as the MDR1 gene (*i.e.*, a nucleic acid encoding MDR1), when the gene has been introduced inside the cell and the coding sequence of the gene is operatively linked to an expression control sequence. The transducing gene is carried by a vector and the gene may or may not be
30 integrated (covalently linked) into chromosomal DNA making up the genome of the cell. A stably transduced cell is one in which the transducing gene has become integrated into a chromosome so that it is inherited by daughter cells through

chromosome replication. This stability is demonstrated by the ability of the cell to establish cell lines or clones comprised of a population of daughter cells containing the transducing gene. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable
5 of stable growth *in vitro* for many generations.

As used herein a "gene-modified hematopoietic stem cell" is a hematopoietic stem cell that has been transduced by a heterologous gene.

10 As used herein the "expansion" of an hematopoietic stem cell indicates that there is an increase in the absolute number of hematopoietic stem cells, *i.e.*, during the culturing of the cells. Analogously, an hematopoietic cell that has undergone such expansion has been "expanded".

15 As used herein "engrafting" a stem cell, preferably an expanded hematopoietic stem cell, means placing the stem cell into an animal, *e.g.*, by injection, wherein the stem cell persists *in vivo*. This can be readily measured by the ability of the hematopoietic stem cell, for example, to contribute to the ongoing blood formation.

20 As used herein an "ABC transporter" is used in the conventional sense and is used to describe a protein that is a transport ATPase. ABC transporters are members of a large family of transport proteins that are ATP-dependent. The name is derived from a highly conserved ATP-binding cassette contained by all of the members. [See, Alberts *et al.*, *Molecular Biology of the Cell*, 3rd edition, Garland Publishing Inc.
25 (New York) Pages 519-522 (1994)]. MDR1 and BCRP are two transmembrane efflux pumps that are part of the family of ABC transporters.

The Breast Cancer Resistance Protein is an ATP transporter protein that contains a conserved ATP-binding cassette which has been isolated from a number of different
30 cell lines and mammalian tissues. Names in the literature of genes encoding this protein include *Bcrp1*, *Mxr*, *Abcp* and ABCG2 gene. Whereas, the murine gene is generally termed as the *Bcrp1* gene and the corresponding human gene has been

termed the ABCG2 gene, as used herein, "BCRP" is meant to include all of such ATP transport proteins obtained from any mammalian source. The murine protein is also referred to herein as mBCRP, whereas the human protein is termed herein, "huBCRP" when the specific mouse or human gene or gene product(s) are being particularly
5 referenced. One huBCRP gene product which is encoded by the nucleotide sequence of SEQ ID NO:9 and has the amino acid sequence of SEQ ID NO:10, whereas another variant is encoded by the nucleotide sequence of SEQ ID NO:26 and has the amino acid sequence of SEQ ID NO:27. One mBCRP is encoded by the nucleotide sequence of SEQ ID NO: 13 and has the amino acid sequence of SEQ ID NO: 14 and another
10 mBCRP is encoded by a nucleotide sequence comprising SEQ ID NO:11 and has an amino acid sequence comprising SEQ ID NO:12.

A "replicon" is any genetic element (e.g., plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication *in vivo*; i.e., capable of replication under its
15 own control.

A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment. The term "vector" can also refer to a recombinant virus or defective virus containing a
20 replicon to which another DNA segment may be attached.

A "DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in its either single stranded form, or a double-stranded helix. This term refers only to the primary and secondary structure of the molecule,
25 and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the
30 nontranscribed strand of DNA (i.e., the strand having a sequence homologous to the mRNA).

A "coding sequence" is a nucleic acid sequence which can be reverse transcribed (*i.e.*, when part of a retroviral vector) and/or transcribed and then translated into a polypeptide *in vitro* and/or *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. A polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

A nucleic acid sequence is "operatively linked" to an expression control sequence when the expression control sequence controls and regulates the transcription and/or translation of that nucleic acid sequence. The term "operatively linked" includes having an appropriate start signal (*e.g.*, ATG) in front of the nucleic acid sequence to be expressed and maintaining the correct reading frame to permit expression of the nucleic acid sequence under the control of the expression control sequence and production of the desired product encoded by the nucleic acid sequence. If a gene that one desires to insert into a recombinant DNA molecule does not contain an appropriate start signal for example, such a start signal can be inserted in front of the gene.

Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for the expression of a coding sequence in a host cell.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined for example, by

mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes.

- 5 A coding sequence is "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then translated into the protein encoded by the coding sequence.

Any of a wide variety of expression control sequences — sequences that control the
10 expression of a DNA sequence operatively linked to it — may be used in these vectors to express the DNA sequences of this invention. Such useful expression control sequences include, for example, the early or late promoters of SV40, CMV, vaccinia, polyoma, adenovirus, herpes virus and other sequences known to control the expression of genes of mammalian cells, and various combinations thereof.

15

In selecting an expression control sequence, a variety of factors will normally be considered. These include, for example, the relative strength of the system, its controllability, and its compatibility with the particular DNA sequence or gene to be expressed, particularly as regards potential secondary structures. Considering these
20 and other factors a person skilled in the art will be able to construct a variety of vector/expression control sequence combinations that will express the heterologous genes used in the present invention.

The phrase "pharmaceutically acceptable" refers to molecular entities and
25 compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human. Preferably, as used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized
30 pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, such as water and

oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or aqueous solution saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solutions.

5

The phrase "therapeutically effective amount" is used herein to mean an amount sufficient to reduce by at least about 15 percent, preferably by at least 50 percent, more preferably by at least 90 percent, and most preferably prevent and/or treat, a clinically significant deficit in the activity, function and response of the host.

10 Alternatively, a therapeutically effective amount is sufficient to cause an improvement in a clinically significant condition in the host.

Nucleic Acid Probes

A nucleic acid molecule is "hybridizable" to another nucleic acid molecule, such as a
15 cDNA, genomic DNA, or RNA, when a single stranded form of the nucleic acid molecule can anneal to the other nucleic acid molecule under the appropriate conditions of temperature and solution ionic strength [see Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook
20 *et al.*, 1989" and Sambrook and Russell, *Molecular Cloning: A Laboratory Manual*, Third Edition (2001) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York]. The conditions of temperature and ionic strength determine the "stringency" of the hybridization. Specific hybridization conditions, corresponding to a T_m of 55°, can be used, *e.g.*, 5x SSC, 0.1% SDS, 0.25% milk, and no formamide; or 30%
25 formamide, 5x SSC, 0.5% SDS. Moderate stringency hybridization conditions correspond to a higher T_m , *e.g.*, 40% formamide, with 5x or 6x SSC. High stringency hybridization conditions correspond to the highest T_m , *e.g.*, 50% formamide, 5x or 6x SSC. These conditions can be used for both annealing and wash steps. Hybridization requires that the two nucleic acids contain complementary sequences, although
30 depending on the stringency of the hybridization, mismatches between bases are possible. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known

in the art. The greater the degree of similarity or homology between two nucleotide sequences, the greater the value of T_m for hybrids of nucleic acids having those sequences. The relative stability (corresponding to higher T_m) of nucleic acid hybridizations decreases in the following order: RNA:RNA, DNA:RNA, DNA:DNA.

- 5 For hybrids of greater than 100 nucleotides in length, equations for calculating T_m have been derived [*see* Sambrook *et al.*, *supra*, 9.50-10.51]. For hybridization with shorter nucleic acids, *i.e.*, oligonucleotides, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity [*see* Sambrook *et al.*, *supra*, 11.7-11.8]. Preferably a minimum length for a hybridizable
- 10 nucleic acid (probe and/or primer) is at least about 12 nucleotides; preferably at least about 18 nucleotides; and more preferably the length is at least about 27 nucleotides; and most preferably at least about 36 nucleotides. Particular primers for *BCRP*, and *mdr1a*, *mdr1b*, and *mdr2* are provided in Example 1 below.
- 15 Such nucleotide probes and primers can be labeled or used to label complementary DNA (where appropriate) by any number of ways well known in the art including using a radioactive label, such as ^3H , ^{14}C , ^{32}P , or ^{35}S , a fluorescent label, a boron label [U.S. Patent No: 5,595,878, Issued January 21, 1997 and U.S. Patent No: 5,876,938, Issued March 2, 1999 which are incorporated by reference in their entireties], and
- 20 enzymatic tags such as urease, alkaline phosphatase or peroxidase. In the case of enzyme tags, colorimetric indicator substrates are known which can be employed to provide a means visible to the human eye or spectrophotometrically, to identify specific hybridization with complementary nucleic acid-containing samples.

25 Antibodies to the ABC Transporters of the Present Invention

- According to the present invention, ABC transporters as produced by a recombinant source, or through chemical synthesis, or an ABC transporter isolated from a natural source; and derivatives or analogs thereof, including fusion proteins, may be used as an immunogen to generate antibodies that recognize an ABC transporter such as
- 30 *BCRP*. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric including humanized chimeric, single chain, Fab fragments, and a Fab expression library.

In a particular embodiment an antibody is raised to an external epitope of BCRP. In a particular embodiment the epitope is derived from the extracellular portion of BCRP. Such an antibody can be used to sort living cells on a flow cytometer. These
5 antibodies can be used, for example, to sort hematopoietic cells based on BCRP (*bcrp*) expression. Such antibodies also may be used to detect BCRP as a marker for repopulating activity.

The anti-BCRP antibodies of the invention may be cross reactive, that is, they may
10 recognize a BCRP derived from a different source, *e.g.*, an anti-human BCRP antibody may recognize both human and mouse BCRP. Polyclonal antibodies have greater likelihood of cross reactivity. Alternatively, an antibody of the invention may be specific for a single form of a BCRP, such as the huBCRP having the amino acid sequence of SEQ ID NO:10.

15 Various procedures known in the art may be used for the production of polyclonal antibodies to BCRP, for example, or derivative or analog thereof. For the production of antibody, various host animals can be immunized by injection with the BCRP, or a derivative (*e.g.*, or fusion protein) thereof, including but not limited to rabbits, mice,
20 rats, sheep, goats, etc. In one embodiment, the BCRP can be conjugated to an immunogenic carrier, *e.g.*, bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH). Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active
25 substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (*bacille Calmette-Guerin*) and *Corynebacterium parvum*.

For preparation of monoclonal antibodies directed toward the BCRP, or analog, or
30 derivative thereof, any technique that provides for the production of antibody molecules by continuous cell lines in culture may be used. These include but are not limited to the hybridoma technique originally developed by Kohler and Milstein

[*Nature*, 256:495-497 (1975)], as well as the trioma technique, the human B-cell hybridoma technique [Kozbor *et al.*, *Immunology Today*, 4:72 (1983); Cote *et al.*, *Proc. Natl. Acad. Sci. USA*, 80:2026-2030 (1983)], and the EBV-hybridoma technique to produce human monoclonal antibodies [Cole *et al.*, in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96 (1985)]. In an additional embodiment of the invention, monoclonal antibodies can be produced in germ-free animals utilizing recent technology [PCT/US90/02545]. In fact, according to the invention, techniques developed for the production of "chimeric antibodies" [Morrison *et al.*, *J. Bacteriol.*, 159:870 (1984); Neuberger *et al.*, *Nature*, 312:604-608 (1984); Takeda *et al.*, *Nature*, 314:452-454 (1985)] by splicing the genes from a mouse antibody molecule specific for a BCRP together with genes from a human antibody molecule of appropriate biological activity can be used; such antibodies are within the scope of this invention. Such human or humanized chimeric antibodies are preferred for use in therapy of human diseases or disorders (described *infra*), since the human or humanized antibodies are much less likely than xenogeneic antibodies to induce an immune response, in particular an allergic response, themselves. In a particular embodiment, the BCRP-expressing cells of the present invention are used to raise monoclonal antibodies to external cell surface epitopes. Antibody producer clones can be screened for differential staining of producer cells versus their parental packaging cells (*see* Figure 1).

According to the invention, techniques described for the production of single chain antibodies [U.S. Patent Nos. 5,476,786 and 5,132,405 to Huston; U.S. Patent 4,946,778] can be adapted to produce for example, BCRP-specific single chain antibodies. An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries [Huse *et al.*, *Science*, 246:1275-1281 (1989)] to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for a BCRP, or its derivatives, or analogs.

Antibody fragments which contain the idiotype of the antibody molecule can be generated by known techniques. For example, such fragments include but are not limited to: the F(ab')₂ fragment which can be produced by pepsin digestion of the

antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the F(ab')₂ fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

- 5 In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art, *e.g.*, radioimmunoassay, ELISA (enzyme-linked immunosorbant assay), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, *in situ* immunoassays (using colloidal gold, enzyme or radioisotope labels, for
10 example), Western blots, precipitation reactions, agglutination assays (*e.g.*, gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, flow cytometry, and immunoelectrophoresis assays, etc. In one embodiment, antibody binding is detected by detecting a label on the primary antibody. In another embodiment, the primary
15 antibody is detected by detecting binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labeled. Many means are known in the art for detecting binding in an immunoassay and are within the scope of the present invention. For example, to select antibodies which recognize a specific epitope of BCRP, one may assay generated hybridomas for a product which
20 binds to the BCRP fragment containing such epitope and choose those which do not cross-react with BCRP. For selection of an antibody specific to a BCRP from a particular source, one can select on the basis of positive binding with BCRP expressed by or isolated from that specific source.
- 25 The present invention also provides methods for producing BCRP monoclonal antibodies that can be used to isolate living stem cells (*see* Figure 1). In a preferred embodiment of this type the living stem cells are isolated by a cell sorting technique. One initial step can be to obtain a cDNA sequence encoding a BCRP and then inserting it into a vector construct (preferably a retroviral vector). This construct can
30 then be introduced into packaging cells (*e.g.*, retroviral packaging cells) to produce transducing vector particles. When GPE86 packaging cells are used, ecotropic ABCG2 (*i.e.*, huBCRP) vector is produced in the supernatant, and can be used to

transduce murine 3T3 cells. After applying the supernatant to logarithmically growing cells, transduced 3T3 cells that express the huBCRP can be isolated by flow cytometry based on their capacity to efflux the fluorescent dye Hoechst 33342. The 3T3 cells which express huBCRP (oval cell, *see* Figure 1), can then be injected into
5 the intraperitoneal space in mice. The strategy of using living cells transduced with the vector increases the probability that the immune system will detect external huBCRP epitopes in their native configuration, rather than epitopes that are internally located in the cells, or epitopes only present in denatured protein.

10 After several rounds of immunization, mice that show anti-huBCRP activity in the peripheral serum can be killed. Their spleens are then isolated and about 500 hybridoma clones are obtained by fusion with myeloma cells using standard techniques. Supernatants from these growing fusion clones are then screened for the presence of anti-huBCRP antibodies. This screening process involves transducing
15 human MCF7 breast cancer cells with the huBCRP vector.

As depicted in Figure 1, the reason that human breast cancer cells are preferred is that unwanted monoclonal antibodies (small cell #3) that react to native 3T3 cell proteins (squares and triangles) are not likely to bind to the human proteins present on MCF7
20 cells. Supernatants are then incubated with the transduced MCF7 cells which express huBCRP. These cells are then stained with a phycoerythrin (PE) conjugated secondary antibody that recognize mouse immunoglobulins. Flow cytometry is then used to detect cell samples that have PE-fluorescence, *i.e.*, cells that bind the murine antibodies from the hybridoma supernatants. These supernatants are likely to contain
25 anti-huBCRP antibodies from their respective monoclonal hybridomas (cell #1). However, it is also possible that the reactivity observed is not due to anti-huBCRP activity, but rather to a murine antibody that cross-reacts with a surface protein found on the MCF7 cell (hybridoma clone 2). To identify and eliminate such clones, all positive supernatants can be back-screened with the parental (non-transduced) MCF7
30 cells. These cells do not express huBCRP, but do express all of the native surface proteins present on the transduced MCF7 cells. Therefore, all clones found to react with the parental (non-transduced) MCF7 cells are discarded.

Hybridoma clones that pass this screening process are then expanded, resubcloned, and reanalyzed. Subclones that pass a second round of screening are then used to make monoclonal antibody containing supernatant in an *ex vivo* roller-bottle
5 production system. The antibody can then be purified on an affinity column, the isotype can be determined, and the concentration of the antibody measured. This preparation can then be used to identify native stem cells that express the huBCRP gene product on their surface, and to isolate such cells by methods including but not limited to flow cytometry, immunoaffinity columns, or magnetic bead procedures.

10

The foregoing antibodies can be used in methods known in the art relating to the localization and activity of the BCRP, *e.g.*, for Western blotting, imaging BCRP *in situ*, measuring levels thereof in appropriate physiological samples, etc. using any of the detection techniques mentioned herein or known in the art. In a specific
15 embodiment, antibodies that agonize or antagonize the activity of BCRP can be generated. Such antibodies can be tested using the assays that measure the drug pumping ability of BCRP, for example.

The antibodies to the ABC transporters can be labeled. Suitable labels include
20 enzymes, fluorophores (*e.g.*, fluorescein isothiocyanate (FITC), phycoerythrin (PE), Texas red (TR), rhodamine, free or chelated lanthanide series salts, especially Eu^{3+} , to name a few fluorophores), chromophores, radioisotopes, chelating agents, dyes, colloidal gold, latex particles, ligands (*e.g.*, biotin), and chemiluminescent agents. In the instance where a radioactive label, such as the isotopes ^3H , ^{14}C , ^{32}P , ^{35}S , ^{125}I , and
25 ^{131}I , are used, known currently available counting procedures may be utilized. In the instance where the label is an enzyme, detection may be accomplished by any of the presently utilized colorimetric, spectrophotometric, fluorospectrophotometric, amperometric or gasometric techniques known in the art.

30 Direct labels are one example of labels which can be used according to the present invention. A direct label has been defined as an entity, which in its natural state, is readily visible, either to the naked eye, or with the aid of an optical filter and/or

applied stimulation, *e.g.* ultraviolet light to promote fluorescence. Among examples of colored labels, which can be used according to the present invention, include metallic sol particles, for example, gold sol particles such as those described by Leuvening (U.S. Patent 4,313,734); dye sol particles such as described by Gribnau *et al.* (U.S. Patent 4,373,932) and May *et al.* (WO 88/08534); dyed latex such as described by May, *supra*, Snyder (EP-A 0 280 559 and 0 281 327); or dyes encapsulated in liposomes as described by Campbell *et al.* (U.S. Patent 4,703,017). Other direct labels include a radionucleotide, a fluorescent moiety or a luminescent moiety. In addition to these direct labeling devices, indirect labels comprising enzymes can also be used according to the present invention. Various types of enzyme linked immunoassays are well known in the art, for example, alkaline phosphatase and horseradish peroxidase, lysozyme, glucose-6-phosphate dehydrogenase, lactate dehydrogenase, urease, these and others have been discussed in detail by Eva Engvall in Enzyme Immunoassay ELISA and EMIT in *Methods in Enzymology*, 70:419-439 (1980) and in U.S. Patent 4,857,453.

Suitable enzymes include, but are not limited to, alkaline phosphatase and horseradish peroxidase. In addition, an antibody can be modified to contain a marker protein such as green fluorescent protein as described in U.S. Patent No. 5,625,048 filed April 29, 1997, WO 97/26333, published July 24, 1997 and WO 99/64592 all of which are hereby incorporated by reference in their entireties. Other labels for use in the invention include magnetic beads or magnetic resonance imaging labels.

In another embodiment, a phosphorylation site can be created on an antibody of the invention for labeling with ^{32}P , *e.g.*, as described in European Patent No. 0372707 (application No. 89311108.8) by Sidney Pestka, or U.S. Patent No. 5,459,240, issued October 17, 1995 to Foxwell *et al.*

Antibodies also can be labeled by metabolic labeling. Metabolic labeling occurs during *in vitro* incubation of the cells that express the protein in the presence of culture medium supplemented with a metabolic label, such as [^{35}S]-methionine or [^{32}P]-orthophosphate. In addition to metabolic (or biosynthetic) labeling with [^{35}S]-

methionine, the invention further contemplates labeling with [^{14}C]-amino acids and [^3H]-amino acids (with the tritium substituted at non-labile positions).

Stem Cell Purification

5 After an appropriate antibody is identified, mouse bone marrow cells, for example, can be depleted of lineage positive cells, and the lin^- cells can be sorted for BCRP expression. Competitive repopulation assays can then be used to demonstrate the enrichment of stem cell activity in the BCRP-expressing fraction. BCRP sorting experiments can also be performed in lin^- , ckit^+ , sca1^+ cells, and in CD34-cells to
10 determine if these populations can be further enriched for stem cell activity. An analogous procedure can be performed using an alternative cell source, *e.g.*, human cord blood cells.

Thus, BCRP sorting experiments can be performed using lin^- , CD34-cells, as well as
15 CD34 $^+$, CD38 $^-$ cells to determine the amount that BCRP expression enriches for repopulating activity in these stem cell populations. Sorted cells then can be injected into NOD/SCID mice in limiting dilution analyses to quantify the stem cell frequencies in these populations. This procedure can be repeated using bone marrow cells and cytokine-mobilized peripheral blood stem cells to demonstrate the utility of
20 the procedure in various clinical stem cell sources. For example, SP cells were isolated from mouse muscle satellite cells and RT-PCR and FACS analysis was used to demonstrate that BCRP is also expressed in these cells (*see* Example 1 below). Reconstitution studies can also be performed using sorted BCRP-expressing muscle cells. Sorted donor cells can be identified after transplant using a GFP-transgenic
25 mouse line for donor cells, for example, and analyzing recipients for GFP $^+$ SP cells in the muscle.

Therefore, the present invention provides a functional basis for identifying SP stem cells, and furthermore, provides a new way to isolate stem cells both for research and
30 clinical applications. For example, the present invention provides a method of isolating stem cells using an anti-BCRP antibody. These stem cells can originate from any tissue that contains stem cells including from bone marrow cells, muscle cells and

even brain cells. Any method that allows the separation of cells that can be distinguished by their ability to bind a particular antibody can be employed. For example, to isolate hematopoietic stem cells, bone marrow cells can be obtained from an animal subject, (preferably a human). Single cell suspensions can then be
5 prepared. An anti-BCRP antibody can be incubated with the cells and the cells can be isolated using standard cell sorting methodology *e.g.*, by fluorescent cell sorting [Bhatia *et al.*, *Nat. Med.* 4:1038-1045 (1998)]. In a related embodiment, muscle stem cells can be isolated from a muscle cell sample [Gussoni *et al.*, *Nature* 401:390 (1999)]. Alternatively, stem cells can be distinguished from non-stem cells by the
10 specificity of the drug-pumping activity of BCRP.

Bone marrow cells can be obtained from any number of sources from an animal, including a human subject. For example, the cells can be harvested from iliac bone marrow from laboratory animals. Alternatively, hematopoietic stem cells can be
15 obtained from umbilical chord cells. Another source for hematopoietic stem cells is from circulating fetal blood cells. In addition, a human subject, for example, can be treated with a cytotoxic drug and/or a hematopoietic stem cell stimulating cytokine (*e.g.*, G-CSF). Mononuclear cells can then be collected by leukaphoresis and the hematopoietic stem cells can be isolated from the peripheral blood cells by their
20 selective binding to an antibody raised against CD34. One source of embryonic stem cells is embryonic/fetal tissue. Sources for obtaining stem cells from a particular tissue type (such as brain or liver) include but are not limited to biopsy samples and cadavers.

BCRP enriched stem cells can be used in the same way, and for the same purposes, as the enriched stem cells that are currently available, *e.g.*, CD34 enriched stem cells. Indeed, the embryonic stem cells provided can be used generally, whereas specific adult stem cells obtained by the methods of the present invention can be used for specific stem cell transplantation *e.g.*, hemopoietic stem cells for bone marrow
25 transplantation, pancreatic stem cells to treat type II diabetes, and brain stem cells to treat Parkinson's disease. The stem cells provided by the present invention can also be used as cell targets in gene therapy protocols. Importantly, cell samples that are
30

identified/selected to be enriched for BCRP expression by the methods disclosed herein comprise a more highly purified population of stem cells than any of the other currently available sources of stem cells that have been isolated based on any alternative stem cell marker (*e.g.*, cell samples selected for expression of CD34).

5 Thus, the enriched stem cell preparations provided by the present invention comprise fewer deleterious anti-host reacting cells and thereby their use significantly lowers the risk of graft *versus* host disease, and/or transplant rejection and may broaden the number of available donors for a given patient.

10 Therefore, the BCRP enriched cell samples of the present invention provide a preferred source of purified stem cells for therapeutic applications. These more highly enriched stem cell samples also allow therapeutic applications of stem cells such as allogeneic transplantation, that heretofore, could not be reliably performed due to the relatively higher level of contaminating non-stem cells in prior stem cell preparations.

15

Furthermore, these highly enriched stem cells are preferred for gene therapy because their use leads to an increase in transduction efficiency and reduces the amount of vector needed for transduction.

20

BCRP Expression and Leukemia

A significant number of leukemic cells, particularly Acute Myelogenous Leukemia (AML) blasts, can efflux fluorescent dyes and certain chemotherapeutic agents such as mitoxantrone and duanomycin, despite the absence of MDR1 and MRP1 expression.

A substantial proportion of these cases are contemplated to be due to the expression of

25 BCRP. AML blasts from newly diagnosed pediatric patients can be assayed for BCRP expression using a variety of techniques as described herein in the context of stem cell identification and purification. Suitable techniques particularly include the use of flow cytometry after staining with a specific anti-BCRP antibody, immunocytochemistry, staining protein lysates with BCRP antibody in Western Blot
30 analyses, and methods that measure BCRP mRNA levels such as PCR, real time PCR, Northern Blot analyses, and ribonuclease protection assays. In cases where significant levels of BCRP expression is found, it can be determined whether the expression of

BCRP is due to mutations in the promoter, hypomethylation of promoter sequences, or due to changes in the transcription factor environment.

Determination of BCRP expression in leukemic cells, particularly AML blasts, provides useful information to the medical practitioner with regard to predicting the efficacy of administration of chemotherapeutic agents which are susceptible to efflux via BCRP. Such information may be used by the medical practitioner to determine the optimal course of treatment for a patient suffering from leukemia, particularly AML. For example, in those cases where the leukemic cells of a patient are determined to be expressing significant levels of BCRP, a course of treatment which does not rely upon agents susceptible to reduced efficacy via BCRP efflux such as bone marrow transplant or immunotherapy may be used. Furthermore, in cases where BCRP expression predicts poor prognosis with standard treatment, more aggressive treatment plans can be implemented at the outset including more aggressive chemotherapy, or bone marrow transplantation approaches.

The present invention may be better understood by reference to the following non-limiting Examples, which are provided as exemplary of the invention. These examples are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed, however, as limiting the broad scope of the invention.

EXAMPLES

EXAMPLE 1

BCRP IS EXPRESSED IN A WIDE VARIETY OF SIDE POPULATION STEM CELLS, BLOCKS DIFFERENTIATION OF TRANSDUCED HEMATOPOIETIC STEM CELLS AND CAN BE USED AS A MARKER FOR PURIFICATION OF STEM CELLS

Introduction

Hematopoietic stem cells (HSCs) can be identified by staining with fluorescent dyes such as Rhodamine (Rho) 123 and purified based on efflux of such dyes [Orlic *et al.*, *Blood* 82:762-770 (1993); Fleming *et al.*, *J.Cell Biol.* 122:897-902 (1993); Spangrude and Johnson, *Proc.Natl.Acad.Sci.USA* 87:7433-7437 (1990); Zijlmans *et al.*, *Proc*

Natl Acad Sci USA 92:8901-8905 (1995)] and Hoechst 33342 [McAlister *et al.*, *Blood* 75:1240-1246 (1990); Leemhuis *et al.*, *Exp.Hematol.* 24:1215-1224 (1996); Wolf *et al.*, *Exp.Hematol.* 21:614-622 (1993)]. The most primitive HSCs are characterized by low degrees of fluorescence after staining with these dyes, a property ascribed to both
5 their capacity for dye efflux, and to relatively low degrees of mitochondrial staining [Kim *et al.*, *Blood* 91:4106-4117 (1998)]. A related method for stem cell identification has been based on Hoechst dye-staining of whole bone marrow cells, followed by dual emission wavelength analysis by flow cytometry. This technique identifies a small fraction of side population (SP) cells that are highly enriched for
10 repopulating activity [Goodell *et al.*, *J.Exp.Med.* 183:1797-1806 (1996)].

This SP population is highly enriched for repopulating cells and is present in the bone marrow of all species examined [Goodell, M.A. *et al.*, *Nat. Med.* 3:1337-1345 (1997)]. The SP phenotype can be blocked by drugs which inhibit cellular dye efflux
15 mechanisms. It has been thought that the efflux activity responsible for the SP phenotype may be due to expression of P-glycoproteins (P-gps), the products of the mammalian multidrug resistance genes (MDR1 in humans and *mdr1a* and *1b* in mice) [Sorrentino, B.P. *et al.*, *Blood* 86: 491-501 (1995)]. This possibility is suggested by the facts that: (i) Rho123 and Hoechst 33342 are substrates for P-gp, (ii) primitive
20 human hematopoietic cells express high levels of P-gp [Chaudhary and Roninson, *Cell* 66:85-94 (1991)], and (iii) the phenotype of SP cells can be blocked by verapamil, a competitive inhibitor of P-gp [Goodell *et al.*, *J.Exp.Med.* 183:1797-1806 (1996)].

25 More recent evidence shows that the muscle contains reconstituting cells that can be identified by the SP phenotype [Gussoni *et al.*, *Nature* 401:390-394 (1999); Jackson *et al.*, *Proc.Natl.Acad.Sci.U.S.A* 96:14482-14486 (1999), *see comments*] suggesting that expression of ABC transporters may be a general stem cell property [Orkin, S.H., *Nat. Med.* 6:1212-1213 (2000)]. Indeed whatever their exact function, it appears that
30 expression of ABC transporters has been evolutionarily conserved in stem cells. The conservation of transporter expression in a wide variety of stem cells is consistent with an important functional role in stem cells.

Although many of the genes encoding ABC transporters were first identified based on their ability to confer drug resistance in tumor cells, it has recently become apparent that they can exert more general effects on cellular function. For example, MDR1
5 gene expression has been shown to inhibit caspase-dependent apoptosis in a variety of cells [Smyth *et al.*, *Proc.Natl.Acad.Sci.USA* 95:7024-7029 (1998)] including hematopoietic cells [Johnstone *et al.*, *Blood* 93:1075-1085 (1999)]. P-gps can also function as lipid translocases by redistributing membrane phospholipids from the inner to outer leaflet of the cell membrane [van Helvoort *et al.*, *Cell* 87:507-517
10 (1996)].

Direct evidence that ABC transporters can have a functional effect in HSCs comes from studies of MDR1 gene transfer in mice [Bunting *et al.*, *Blood* 92:2269-2279 (1998); Example 1 of U.S. Serial No. 09/584,586 filed May 31, 2000, the disclosure
15 of which are hereby incorporated by reference in their entireties]. When murine bone marrow cells were transduced with an MDR1-expressing retroviral vector, dramatic expansion of repopulating stem cells was noted during a 12-day culture period [Bunting *et al.*, *Blood* 92:2269-2279 (1998); Example 1 of U.S. Serial No. 09/584,586
filed May 31, 2000, the disclosure of which are hereby incorporated by reference in
20 their entireties]. In contrast, repopulating activity was lost over time in control cultures [Bunting *et al.*, *Blood* 96:902-909 (2000); Example 2 of U.S. Serial No. 09/584,586 filed May 31, 2000, the disclosure of which is hereby incorporated by
reference in its entirety]. These results demonstrate that enforced expression of MDR1 results in stem cell self-renewal and expansion during extended culture
25 periods. This expansion of repopulating cells was associated with a parallel increase in SP cells, while SP cells were lost over time in control cultures. These results directly link ABC transporter expression, or at least MDR1 expression, with the SP stem cell phenotype.

30 One possible mechanism for the stem cell expansion was that P-gp expression could result in the efflux of toxic media components from HSCs during the *ex vivo* culture period. This possibility was ruled out by the observation that MDR1-transduced stem

cells had a direct proliferative advantage *in vivo* [Example 2 of U.S. Serial No. 09/584,586 filed May 31, 2000, the disclosure of which is hereby incorporated by reference in its entirety]. When transduced bone marrow cells were directly transplanted in irradiated mice, without an *ex vivo* expansion phase, there was a progressive outgrowth of MDR1-transduced cells relative to a control graft. These results show that MDR1 expression was conferring a more general effect on stem cell division, and not simply acting through a detoxification mechanism specific to *ex vivo* culture. Experiments with a mutant P-gp construct demonstrated that HSC expansion required the molecular pump function of P-gp, suggesting that the mechanism of expansion involved modulation of some endogenous molecular substrate within HSCs [Example 2 of U.S. Serial No. 09/584,586 filed May 31, 2000, the disclosure of which is hereby incorporated by reference in its entirety]. Collectively, these studies show that MDR1 gene expression can promote HSC self-renewal and amplification. Heretofore, it was not known if this property is unique to the MDR1 gene, or whether other ABC transporters can exert a similar function.

Results

The regulated expression of endogenous P-gps in HSCs appears to be important in facilitating the self-renewal divisions that maintain the stem cell compartment over time [see Examples 1 and 2 of U.S. Serial No. 09/584,586 filed May 31, 2000, the disclosure of which is hereby incorporated by reference in its entirety] and indeed, one or more naturally occurring endogenous ABC transporters apparently plays a critical functional role in stem cell homeostasis. This premise is consistent with two observations: (i) HSCs universally express dye-effluxing transporters; and (ii) enforced expression of MDR1 leads to stem cell amplification and myeloproliferation [see Example 1 of U.S. Serial No. 09/584,586 filed May 31, 2000, the disclosure of which is hereby incorporated by reference in its entirety].

As disclosed herein, an alternate ABC transporter(s) is expressed in SP stem cells derived from the *mdr1a/1b* knockout mouse. The analyses of mRNA from sorted SP stem cells have identified several newly cloned transporters that are expressed in SP HSCs, that may possibly have a role in the self-renewal process of HSCs. The most

highly expressed is the *Bcrp1/Mxr/Abcp/ABCG2* gene product, BCRP. Importantly, there is no detectable expression of BCRP in peripheral blood leukocytes, spleen, or thymus at the level of Northern blot analysis, while small but detectable amounts of BCRP mRNA were expressed in human fetal liver.

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An ABC transporter other than P-gp is expressed in murine SP HSCs: Quantitative repopulation assays were performed using commercially available *mdr1a/ab*^{-/-} mice as donors because it is well known that severe quantitative stem cell abnormalities can coexist with relatively normal peripheral blood counts such as in *W/W^v* mice. Normal numbers of repopulating cells were found to be present in the bone marrow. Bone marrow cells were then analyzed for the content of SP cells after staining with Hoechst dye. To confirm that the knockout mice had the expected phenotype, it was verified that the capacity for Rho 123 efflux had been lost in peripheral blood leukocytes as has been previously described [Schinkel *et al.*, *Proc Natl Acad Sci USA* 94:4028-4033 (1997)]. Despite this loss of P-gp related transporter function, SP cells were present in normal numbers in the bone marrow when compared to wild type mice of the same strain. This indicates that another ABC transporter is likely being expressed, potentially compensating for the loss of P-gp function.

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To further test this possibility, biochemical studies were performed on knockout bone marrow cells using known inhibitors of ABC transporter efflux function. Cells were treated with either verapamil or 2-deoxyglucose. Verapamil is a competitive inhibitor of several known ABC transporters including MDR1, whereas 2-deoxyglucose is an inhibitor of ATP synthesis that depletes cellular ATP levels required for ABC transporter function. Treatment with either of these compounds before and during Hoechst 33342 staining eliminated phenotypically identifiable SP cells. These results conclusively demonstrate that another ABC transporter(s) is being expressed in SP cells from the bone marrow of *mdr1a/1b* knockout mice and is (are) responsible for the SP phenotype.

Identification of Bcrp as an expressed ABC transporter in hematopoietic stem cells:

An RT-PCR assay was developed to detect mRNA expression of other known ABC transporters in murine bone marrow SP cells. Based on the published human sequences for MRP1 [Cole *et al.*, *Science* 258:1650-1654 (1992), *see comments*],
5 MRPs 2, 3, 4 [Kool *et al.*, *Cancer Res.* 57:3537-3547 (1997)], and BCRP [Doyle *et al.*, *Proc.Natl.Acad.Sci.U.S.A* 95:15665-15670 (1998);published erratum appears in *Proc Natl Acad Sci U S A*; 96(5):2569 (1999)] homologous sequences from the murine EST database were identified to design PCR primers for cDNA amplification. Multiple primer sets were tested using mouse liver cDNA as a template, and primer
10 sets were chosen that gave specific bands of the expected size. Using FACS, bone marrow SP cells were sorted from both normal mice and the *mdr1a/1b* knockout mouse. Total cellular RNA was prepared from 50,000 and 100,000 purified SP cells, and then used for RT-PCR analysis. These experiments showed that the *Bcrp1* (mBCRP) mRNA was the most highly expressed of all the transporters studied.
15 Moderate expression levels were observed for *mrp4* and *mrp1*, while *mrp3* was expressed at very low levels, and no detectable expression of *mrp2* was observed. The low levels of expression of *mrp1* in the liver correlated with previously described low levels of expression of MRP1 in human liver [Kool *et al.*, *Cancer Res.* 57:3537-3547 (1997)]. Virtually identical results were obtained using sorted SP cells from normal
20 mice.

It is important to note that while SP cells are highly enriched for repopulating cells, at least 250 SP cells are required to achieve significant repopulation in mice [Goodell *et al.*, *J.Exp.Med.* 183:1797-1806 (1996)] indicating that most SP cells are not true stem
25 cells. In contrast, it has previously been shown that CD34⁻, c-kit⁺, Sca1⁺, lineage negative (CD34-KSL) cells from the bone marrow are a relatively pure subset of repopulating cells, with repopulation in about 20% of mice that are transplanted with single sorted cells [Osawa *et al.*, *Science* 273:242-245 (1996)]. Therefore, transporter expression was studied in the highly purified CD34-KSL population, as well as from a
30 number of other different sorted populations.

Like SP cells, the CD34-KSL cells expressed relatively high levels of mBCRP mRNA, however in contrast to SP cells, there were little to no expression of the other ABC transporters. In the more differentiated CD34⁺ KSL cell fraction, there was marked downregulation of mBCRP expression with the appearance of significant expression of *mrl1*, 2, and 4. The S⁺K⁺Lin⁻ population is a mixture of CD34⁺ and + cells, and gave results that were intermediate between the CD34⁺ and - subfractions. mBCRP expression was not detectable in granulocytes, macrophages, B cells, or thymocytes. The only other cell populations with detectable mBCRP expression were erythroid progenitors (Ter119⁺) and natural killer cells (NK1.1⁺). These results suggest that mBCRP expression is highly specific for repopulating stem cells in the lineage negative compartment of the bone marrow, and that expression of other transporters in the sorted SP cell population were likely due to the presence of more differentiated cells with lesser degrees of repopulation potential. These data indicate that BCRP expression should be a useful marker for stem cell identification and purification. The expression data are also consistent with a necessary functional role for BCRP gene expression in repopulating hematopoietic stem cells, and perhaps in SP stem cells from muscle and other tissues.

High levels of expression of BCRP mRNA in sorted SP cells from Rhesus Monkey bone marrow: To determine if primate SP cells were expressing BCRP, a bone marrow aspirate sample was obtained from a normal Rhesus Monkey. After lysis of the red blood cells, the leukocyte population was stained with Hoechst dye and analyzed by flow cytometry for SP cells. The flow pattern was very similar to that obtained with mouse bone marrow, with about 0.05% of cells falling into the SP gate (see above). Sorting was performed and resulted in isolation of 2000 SP cells, and 10,000 cells from a distinct gate outside of the SP region (non-SP cells). RNA was extracted, and a RT-PCR cycle curve using β -actin primers as an internal control showed roughly equivalent signals at 35 cycles for non-SP cells versus 60 cycles for SP cells. These PCR conditions were repeated using BCRP-specific primers in place of the β -actin primers. A strong signal was obtained with BCRP primers at 60 cycles in the SP cells sample, and a much fainter signal was detected at 50 cycles. No BCRP signal was detected at 35 cycles in the non-SP sample, which was the highest cycle

number used for this sample. These results demonstrate relatively specific, high level expression of BCRP mRNA in monkey SP cells since the β -actin signal for non-SP cells at 35 cycles was actually greater than the signal for SP cells at 60 cycles. Taken together with the mouse data, (above) these results show that the expression of a

5 BCRP transporter ortholog is conserved in SP stem cells from diverse species. In addition, these results further confirm that human stem cells can be identified and/or purified by monitoring/exploiting their unique BCRP expression .

Expression of Bcrp1 in murine myoblast SP cells and murine ES cells: Stem cells

10 bearing the SP phenotype have also been identified in murine muscle, and appear to be related to the satellite cells that are located on the periphery of the muscle fiber. Consistently, these cells also appear to be associated with muscle regeneration.

SP cells were therefore isolated from the murine muscle, and assayed for mBCRP

15 expression by RT-PCR to further correlate *Bcrp1* (mBCRP) expression with the SP phenotype. Muscle tissue was dissected, minced, digested with collagenase, and a single cell suspension was stained with Hoechst dye for SP cell analysis. An SP population of cells was observed with FACS analysis that bears a striking resemblance to the profile seen with bone marrow cells. Gated myoblast SP cells

20 were sorted, and RNA was prepared from a fraction of 20,000 cells. RT-PCR analysis showed relatively high levels of mBCRP expression. However, unlike the results with monkey bone marrow, a distinct non-SP cell fraction was not available for analysis. These results further support the conclusion that mBCRP expression can be used to identify SP stem cells from a variety of organs.

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In contrast to the results described above, the non-SP population from murine ES cells expressed *mBCRP* at equivalent levels to the SP fraction. This is not surprising because ES cells are clonally derived and are expected to be homogenous with regard to gene expression. These results show that *mBCRP* expression is conserved in all SP

30 populations that were studied, and suggests a possible required role for mBCRP in stem cell function.

Vector-mediated expression of ABCG2 (huBCRP) directly confers the SP phenotype.

- A pcDNA-based BCRP vector was used to transfect Soas2 cells, which were then analyzed for their dye efflux properties. Transfected clones readily effluxed Hoechst dye but not rho, and the efflux activity was fully inhibited by reserpine. Furthermore,
- 5 an inactivating mutation in the Walker A motif abolished the Hoechst dye efflux activity, showing the requirement for transporter pump function in dye efflux. These findings are fully consistent with the Hoechst low, rho bright phenotype of bone marrow SP cells from *mdr1a/1b*^{-/-} mice.
- 10 A retroviral producer cell line was then used based on the Harvey murine sarcoma virus backbone (HaBCRP) to transduce normal murine bone marrow cells. After culture in myeloid cytokines for 12 days, vector-transduced cells showed a large increase in the number of SP cells, with greater than 60% of the cells falling within the SP region. By comparison, only 0.05% of cells that were mock-transduced
- 15 showed the SP phenotype. HaBCRP-transduced bone marrow cells were also transplanted into lethally irradiated recipients to determine if SP expansion could be directly obtained *in vivo*. Three mice were killed 5 weeks after transplant, and all showed increased proportions of SP cells in the bone marrow ranging between 1.4 to 11.4 % SP cells. Altogether, these results show that expression of HuBCRP
- 20 transporter can directly confer the SP phenotype in transduced primary bone marrow cells.

Enforced expression of HuBCRP in murine bone marrow cells blocks differentiation.

- To determine the functional effects of enforced HuBCRP expression,
- 25 HaBCRP-transduced bone marrow cells were assayed for their ability to generate myeloid progenitor colonies. In cells that were analyzed directly after transduction, a significant decrease in CFU-C and CFU-S formation was seen in HaBCRP-transduced populations. The plating efficiency of CFU-C was decreased between 4-6 fold in 2 independent experiments. Control cells gave confluent CFU-S at a dose of 1×10^5
- 30 cells, while HaBCRP-transduced cells gave only a few discrete colonies. To control for non-specific effects of HuBCRP expression in the producer cells during the coculture phase of transduction, bone marrow cells were also co-cultured with

HaBCRP-transduced 3T3 cells. No effects on progenitor content were seen with this control.

Lethally irradiated C57Bl/6J mice were then transplanted with HaBCRP-transduced
5 cells and the effects on hematopoietic reconstitution were studied. Despite
amplification of SP cells in the bone marrow in all 3 cases, transduction with
HaBCRP was associated with a significant decrease in the peripheral white blood cell
count at 5 weeks, and a parallel decrease in the CFU-C content in the bone marrow.
Competitive repopulation assays were next performed to more precisely quantify the
10 effects of vector expression on myeloid maturation. Mock-transduced bone marrow
cells were competed in equal ratios with either HaBCRP-transduced cells, or with
cells transduced with a control MSCV-GFP vector. At 9 weeks after transplant,
peripheral contributions to the erythrocyte compartment were measured using an
electrophoresis assay that distinguishes between donor specific hemoglobin
15 polymorphisms [Bunting, K.D. *et al.*, *Blood* 96: 902-909 (2000); U.S. Serial No.
09/584,586 filed May 31, 2000, the disclosures of which are hereby incorporated by
reference in their entireties]. These studies showed that the HaBCRP vector caused a
marked inhibition of erythroid repopulation that was not seen in mock controls.
Altogether, these results show that enforced expression of HuBCRP can block
20 hematopoietic differentiation, and suggest that endogenously expressed BCRP
HuBCRP may function to maintain stem cells in a primitive, pluripotent state.

*Derivation of murine monoclonal antibodies against the human ABCG2 (huBCRP)
gene product:* A human BCRP cDNA was obtained as a full length EST from
25 Genome Systems in St. Louis, MO (*see* Methods above). This cDNA was cloned into
the Harvey murine sarcoma virus backbone to create the HaBCRP retroviral vector, as
described above. This vector was next introduced into the ecotropic packaging cell
line GPE86, and vector-containing supernatant was used transduce NIH 3T3 cells. A
polyclonal population of cells (designated 3T3-BCRP) was isolated by flow
30 cytometry, gating on cells that efflux the fluorescent dye Hoechst 33342. Expression
of the HuBCRP gene product in these cells was confirmed by Western blot analysis
using a polyclonal serum raised in rabbits to an internal peptide epitope.

The 3T3-BCRP cells were used to immunize mice. Twenty BALB-C mice were immunized with whole, living 3T3-BCRP cells by injecting 4 million cells directly into the peritoneal space. Fourteen days later, these cells were reinjected as an immunization boost. Individual mice that showed antibody reactivity in the serum were killed and hybridoma clones were isolated after cell fusion and selection with HAT media. Supernatants from each hybridoma clone were screened by flow cytometry using a human breast cancer cell line (MCF-7) that had been transduced with an amphotrophic HaBCRP vector. Any supernatant that showed reactivity in this assay was then back-screened on the parental MCF-7 line, and clones that reacted with the MCF-7 HaBCRP cells but not with the parental MCF-7 line were scored as positive and specific. These cells were then subcloned, and re-screened based on the indicator cell lines (*see* Figure 1).

Independent subclones that show relatively large shifts with the MCF-7 HaBCRP cells, but not with the parental control cells are then isolated. Clones that can detect expression of the HaBCRP vector in bone marrow cells from previously transplanted mice are then expanded to produce larger quantities of supernatant *ex vivo* with a rollerbottle production system. The antibodies are then purified on an affinity column. These antibodies can then be tested for their ability to detect the endogenously expressed huBCRP gene product in human umbilical cord blood samples. Antibodies that detect the endogenously expressed ABCG2 gene product in human umbilical cord blood samples can then be used to identify and/or isolate stem cells.

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Discussion

While Hoechst and rho dye staining have long served as methods for hematopoietic stem cell purification, the molecular basis for this phenotype has not been defined. The results provided herein show that both *Bcrp1* and MDR1 homologues are expressed in a relatively restricted manner in primitive stem cells. The fact that SP cells are not diminished in *mdr1a/b*^{-/-} mice is explained by the compensatory Hoechst efflux activity provided by *Bcrp1* expression. The lack of rho dull SP cells in

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mdr1a/b^{-/-} mice is similarly explained by the fact that Pgp can efflux rho but the BCRP pump cannot. The finding that these dye efflux activities are molecularly distinguishable has significant implications for stem cell purification strategies. For instance, murine ES cells do not efflux rho, implying that Hoechst dye efflux
5 (mBCRP expression) is a more specific stem cell marker.

Several aspects of the data disclosed herein indicate that BCRP plays an important functional role in stem cells. The conserved expression of this transporter in SP stem cells from a wide variety of sources is consistent with a required function. Secondly,
10 the tightly regulated expression pattern of *Bcrp1* in murine hematopoiesis, where expression is markedly decreased in early stages of differentiation, is consistent with a stem cell-specific role. Further evidence is provided by overexpression studies, where enforced huBCRP expression leads to a defect in hematopoietic differentiation. *In vivo*, the HaBCRP vector caused an accumulation of SP cells in the bone marrow, but
15 mature progeny from these cells were significantly decreased in the bone marrow and peripheral blood, and a direct inhibitory effect on CFU-C and CFU-S formation was observed. Altogether, these results are best explained by a model where *Bcrp1* expression in stem cells confers relative resistance to commitment and differentiation and thereby contributes to maintaining a primitive, pluripotent state. This potential
20 anti-differentiative effect would then be alleviated with the observed decrease in expression that occurs during early commitment. Along these lines, the results of enforced expression can now be explained by promiscuous expression of *Bcrp1* in more mature compartments where endogenous expression does not normally occur. The block to development could occur in these compartments as the result of
25 deregulated *Bcrp1* expression.

In considering the mechanism for these effects, it is notable that the normal physiologic functions of BCRP have not yet been defined. The gene was first isolated from human tumor cell lines and shown to be involved in drug resistance [Doyle, L.A.
30 *et al.*, *Proc. Natl. Acad. Sci. U. S. A* 95:15665-15670 (1998); Miyake, K. *et al.*, *Cancer Res.* 59:8-13 (1999); Maliepaard, M. *et al.*, *Cancer Res.* 59: 4559-4563 (1999)]. The primary structure of huBCRP shares the highest homology with the *Drosophila white*

gene product. These "half transporters" have only one ATP binding cassette, and are thought to require homo- or heterodimerization for ATP hydrolysis and function. The *white* gene product heterodimerizes with two other half transporters, and these interactions specifically define eye color in *Drosophila* [Ewart, G.D. *et al.*, *J. Biol. Chem.* 269,10370-10377 (1994)]. BCRP may also form heterodimers in mammalian cells with other as yet undefined partners, and these interactions could affect substrate specificity.

While the mechanism for the effects of huBCRP expression on hematopoietic differentiation is not known, several general possibilities can be proposed. There is a precedent for the ability of ABC transporters to maintain stem cell primitivity in lower organisms. *Dictyostelium* cells express a transport activity defined by rho efflux that maintains cells in a primitive undifferentiated state [Good, J.R. & Kuspa, A., *Dev. Biol.* 220: 53-61 (2000)]. This effect is conferred by efflux of a differentiation-inducing factor, DIF-1, from the interior of prespore cells. It is possible that *Bcrp1* is modulating an analogous substrate in mammalian cells. A second possibility is that the *Bcrp1* pump may play a role in mediating extracellular signals that dictate stem cell interactions with the microenvironment. This type of effect on extracellular signaling has recently been demonstrated in *mrp1*⁺ mice, where this transporter has been shown to be required for normal dendritic cell migration via transport of leukotrienes to the extracellular space [Robbiani, D.R. *et al.*, *Cell* 103:757-768 (2000)]. A third possibility is that *Bcrp1* may be providing a function that is redundant with *mdr1a*, so that the loss of a potential anti-apoptotic signal in stem cells in the *mdr1a/1b*⁺ knockout mice could be compensated by *Bcrp1* expression.

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Methods

Retroviral-mediated gene transfer into murine hematopoietic stem cells: Bone marrow cells can be harvested from C57BL/6 or B6.Ch-1/By (referred to as "HW80") congenic mouse strains (Jackson Laboratories, Bar Harbor, ME) by standard methods. Following isolation, cells are placed into liquid suspension culture in Dulbecco's Modified Eagle's Medium (DMEM) (BioWhittaker, Walkersville, MD) with 1% penicillin/ streptomycin (Gibco/BRL, Grand Island, NY), 15% fetal bovine

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serum (FBS; Hyclone, Logan UT), 20 ng/ml murine interleukin (IL)-3 R & D Systems, Minneapolis, MN), 50 ng/ml human IL-6 (Amgen, Thousand Oaks, CA), and 50 ng/ml murine stem cell factor R & D Systems). The cells are initially plated at 1×10^6 cells/ml in 10 mls of medium. Following pre-stimulation for 48 hours, cells are replated onto confluent monolayers of irradiated ecotropic producer cell lines. The bone marrow cells were plated at the same density used in the pre-stimulation phase and in the same medium with 6 μ g/ml polybrene added. Co-culture with producer cells is continued for 48 hours followed by harvest of bone marrow cells. A small sample of bone marrow cells are plated into methylcellulose to score drug-resistant myeloid progenitors.

Southern Blot Analysis: DNA can be prepared as previously described [Sorrentino *et al.*, Science 257:99-103 (1992)]. Typically 10 to 20 mg of genomic DNA is restriction digested with either EcoRI or NheI, and separated on a 1% agarose gel. Gels can be blotted overnight onto Hybond N⁺ nylon membrane (Amersham), UV crosslinked, and hybridized with either MDR1 or hemoglobin-specific [³²P]-labeled probes. Blots are washed extensively at 65°C, exposed overnight, and can be analyzed on a phosphorimager (Molecular Dynamics).

Rhodamine 123 (Rho123) staining: Rho123 staining can be performed by trypsinizing cells, resuspending the cells in DMEM medium containing 10% FCS at a concentration of 1×10^6 cells/ml, and adding Rho123 (Sigma) at a final concentration of 1 μ g/ml. The cells are then incubated at 37°C for one hour in the dark, washed once with 10 mls of PBS, and resuspended in DMEM/10% FCS. The cells are next incubated at 37°C for one hour to allow for efflux, spun down, and then resuspended in 1 ml of PBS for FACS analysis.

4E3 antibody and Rhodamine 123 staining: Producer cells can be analyzed for an ABC transporter, *e.g.*, P-gp expression by staining with a monoclonal mouse anti-human P-glycoprotein antibody (clone 4E3, DAKO, Carpinteria, CA). Adherent cells are trypsinized, and resuspended in 50 μ l PBS containing 2% BSA and 0.1% NaN₃. 5 μ l of the 4E3 antibody are then added, incubated at room temp (RT) for 30

minutes, washed twice with phosphate buffered saline (PBS), and then resuspended in 50 ul PBS containing 2% BSA and 0.1% NaN₃. After the primary antibody staining, 5 ul of PE-conjugated, rabbit anti-mouse antibody (DAKO) is added as a secondary stain. The cells are then incubated at RT for 30 minutes, washed twice with PBS,
5 resuspended in PBS for FACS analysis.

Hoechst 33342 SP cell assay: Murine bone marrow cells are collected and resuspended at 1×10^6 cells/ml in DMEM plus 10 mM HEPES and 2% FBS. In a water bath, the cells are allowed to equilibrate at 37°C, followed by addition of 5
10 µg/ml Hoechst 33342 (Fisher Scientific, Pittsburgh, PA) for 90 minutes as previously described [Goodell *et al.*, *J. Exp. Med.*, 183:1797-1806 (1996)]. Cells are then centrifuged at 4°C and resuspended in ice cold HBSS plus 10 mM HEPES and 2% FBS at 1×10^7 cells/ml. For flow cytometric analysis or sorting, a Becton Dickinson FACS Vantage flow cytometer (Becton Dickinson, San Jose, CA) can be configured
15 for dual emission wavelength analysis as previously described [Goodell *et al.*, *J. Exp. Med.*, 183:1797-1806 (1996)]. Cells are gated based on forward and side light scatter to exclude debris. Propidium iodide staining (2 µg/ml) can be utilized to derive a gate excluding dead cells. Cells are analyzed at approximately 5,000 cells/second until data from 1×10^6 cells are collected. The SP cell gate can be defined based on normal
20 fresh bone marrow cells, (C57BL/6 for example).

Mice: Both male and female *mdr1a/1b*^{-/-} mice, backcrossed onto the FVB background, were purchased from Taconic Labs (Germantown, NY). For transplant studies, female C57BL/6J and B6.C-H1b/ByJ mice (referred to as HW80) were purchased
25 from Jackson Laboratories (Bar Harbor, ME), and used between 8 and 14 weeks of age.

SP cell analysis. Murine and Rhesus monkey SP cell analyses was performed exactly as previously described [Goodell, M.A. *et al.*, *J. Exp. Med.* 183:1797-1806 (1996);
30 Goodell, M.A. *et al.*, *Nat. Med.* 3:1337-1345 (1997)]. For the inhibitor experiments, 2-deoxyglucose and sodium azide (Sigma, St. Louis), or reserpine (Sigma) were added to cells at a final concentration of 50 and 15 mM, or 5 µM respectively. Cells were

then incubated at 37°C for 15 minutes prior to adding Hoechst 33342 dye. In other experiments, bone marrow cells were incubated in the presence of 0.1 µg/ml rhodamine 123 (Sigma) at 37°C for 20 minutes, then resuspended in medium containing Hoechst 33342 for SP analysis.

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Cell sorting and RNA extraction. Using a FACS Vantage (Becton Dickinson, San Jose, CA) for flow cytometric sorting, approximately 300,000 and 160,000 SP cells were isolated from the bone marrow of *mdr1a/1b*^{-/-} and wildtype mice respectively.

Rhesus monkey bone marrow cells from a 2 ml aspirate were sorted by flow

10 cytometry, and approximately 2,000 SP and 10,000 non-SP cells were isolated. Single cell suspensions of murine skeletal muscle cells were prepared for SP analysis as previously described [Gussoni, E. *et al.*, *Nature* 401:390-394 (1999)], and 10,000 SP and non-SP cells were sorted. The procedure for purifying murine CD34⁺ stem cells has been previously described [Osawa, M. *et al.*, *Science* 273:242-245 (1996)]. In all
15 cases, total RNA from the sorted cells was isolated using RNA STAT-60 (Tel-Test, INC. Friendswood, TX) according to the manufacture's recommendations.

RT-PCR assays. The primers used for PCR amplification are as follows:

20 murine bcrp1: 5'-CCATAGCCACAGGCCAAAGT-3', SEQ ID NO:17 and
5'-GGGCCACATGATTCTTCCAC-3', SEQ ID NO:18 for a 327bp fragment;

Rhesus BCRP: 5'-GGCCTCAGGAAGACTTATGT-3', SEQ ID NO:19 and
5'-AAGGAGGTGGTGTAGCTGAT-3', SEQ ID NO:20 for a 342bp fragment.

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Multiplex PCR for MDR1 like genes utilized a forward primer for *mdr1a* and *mdr2*

5'-AGCTGGAGAGATCCTCACC-3', SEQ ID NO:21,

a forward primer for *mdr1b*

5'-AGCCGGAGAGATCCTCACC-3', SEQ ID NO:22 and

30 reverse primers for *mdr1a*, *mdr1b*, and *mdr2* respectively:

5'-CTGTAGCTGTCAATCTCGGG-3', SEQ ID NO:23,

5'-CTGTAGCTGTCAATCTCAGG-3', SEQ ID NO:24, and

5'-CTGTAGCTGTCAATCAGAGG-3', SEQ ID NO:25 .

These primers amplify a 730bp fragment for all 3 cDNAs.

- 5 PCR products were digested with a mixture of BstXI, BglII, and EcoRI at 37°C to distinguish the relative contributions from each cDNA species (*mdr1a* BstXI digestion: 619 + 111bp, *mdr1b* BglII digestion: 305 + 425bp, *mdr2* EcoRI digestion: 452 + 278bp).
- 10 RNA was treated with RQ1 RNase-free Dnase (Promega, Madison, WI) and reverse transcribed into cDNA by using Superscript II RT (Gibco-BRL, Gaithersburg, MD) and a mixture of oligo dT primers and random hexamers, according to the manufacturers instructions. All cDNAs were first normalized using GAPDH as a
- 15 standard. In the experiments involving murine SP populations, ABC transporter amplification were performed using 32 cycles. In CD34⁺ experiments, GAPDH normalized cDNA aliquots were amplified with mBCRP primers for 35 cycles. In the Rhesus monkey experiments, RNA from 2000 SP cells was amplified for HuBCRP or β -actin using 60 cycles, while RNA from 10,000 non-SP cells was amplified using 35
- 20 cycles, based on the observation that these different conditions gave equivalent β -actin signals from each sample. All PCR reactions were performed using 0.2 μ L P³²-labeled CTP at 800 μ Ci/mmol/L in the reaction mix. After amplification, 20 μ L of the reaction mix was electrophoresed on a 5% nondenaturing polyacrylamide gel. HuBCRP expression vectors, transduction, and transplants.
- 25 The human BCRP cDNA was obtained (Genome Systems, St. Louis, MO) as a full length EST # 52176, SEQ ID NO:26. This cDNA was cloned either into the pcDNA₃ expression vector (Invitrogen, Palo Alto, CA), or used to replace the MDR1 cDNA in the HaMDR1sc vector [Bunting, K.D. *et al.*, *Blood* 92: 2269-2279 (1998); Bunting, K.D. *et al.*, *Blood* 96: 902-909 (2000); U.S. Serial No. 09/584,586 filed May
- 30 31, 2000, the disclosures of which are hereby incorporated by reference in their entirety]. Polyclonal populations of ecotropic producer cells were generated by transducing GP⁺E86 packaging cells [Markowitz, D. *et al.*, *J. Virol.* 62:1120-1124

(1988)] with a transient supernatant derived from transfected 293T cells, as previously described [Persons,D.A. *et al.*, *Blood Cells Mol. Dis.* 24: 167-182 (1998)].

Vector-transduced producer cells were then isolated by cell sorting for Hoechst 33342 dim cells. The methods for transduction of bone marrow cells, *in vitro* expansion of
5 cells, murine transplants, and competitive repopulation assays has been previously described [Allay,J.A. *et al.*, *Nat. Med.* 4:1136-1143 (1998)]. CFU-C and CFU-S assays were done as previously described [Allay,J.A. *et al.*, *Blood* 90: 3546-3554 (1997); Persons,D.A. *et al.*, *Blood* 90:1777-1786 (1997)].

10

EXAMPLE 2

BCRP EXPRESSION IN BLASTS FROM PEDIATRIC ACUTE MYELOGENOUS LEUKEMIA (AML)

Clinical evidence shows an association between dysregulated ABC transporter
15 expression and human leukemia. In chronic myelogenous leukemia, about 60% of chronic phase patients exhibit P-gp expression in leukemic cells from the bone marrow [Giles *et al.*, *Cancer* 86:805-813 (1999)]. In patients with acute myelogenous leukemia (AML), between 35 and 70% of cases demonstrate P-gp expression in leukemic blasts at diagnosis, and P-gp expression was a strongly negative prognostic
20 factor [Leith *et al.*, *Blood* 94:1086-1099 (1999)]. In contrast, normal late myeloid cells in humans do not express P-gp [Drach *et al.*, *Blood* 80:2729-2734 (1992), *see* comments]. In some cases, increased P-gp expression in blast cells was due to hypomethylation of sequences in the MDR1 promoter [Nakayama *et al.*, *Blood* 92:4296-4307 (1998)]. Expression of other ABC transporters can occur in human
25 AML, as has been documented for the multidrug resistance protein (MRP1), and the lung resistance protein (LRP) [Leith *et al.*, *Blood* 94:1086-1099 (1999); Michieli *et al.*, *Br.J.Haematol.* 104:328-335 (1999)]. Importantly, a significant number of AML cases showed inhibitable dye efflux activity that was not associated with MDR1, MRP1, or LRP [Michieli *et al.*, *Br.J.Haematol.* 104:328-335 (1999); Leith *et al.*,
30 *Blood* 86:2329-2342 (1995)].

To determine if BCRP expression could be detected in leukemia cells from pediatric patients with AML, RT-PCR analysis was performed on RNAs derived from leukemic bone marrow from four individual cases. Two cases were strongly positive for BCRP mRNA, with BCRP-amplified fragments detected at 25 cycles of amplification.

- 5 Interestingly both of these "high-expressing" cases were associated with the M1 FAB phenotype. Two other cases with an M5 phenotype did not show any detectable signal at 25 cycles, but BCRP could be detected at 30 cycles. β -actin controls demonstrated that this variation was not due to differences in mRNA loading. The low level signal seen in the M5 cases could have been due to contaminating erythroid progenitors
- 10 which may be expressing BCRP, or due to low level BCRP expression in the blast cells. These data confirm that BCRP mRNA expression can be detected in at least some primary AML samples. Furthermore, these results provide further impetus for using an anti-BCRP antibody to probe blast cell samples in the diagnosis/prognosis of AML.

15

- While the invention has been described and illustrated herein by references to the specific embodiments, various specific material, procedures and examples, it is understood that the invention is not restricted to the particular material combinations of material, and procedures selected for that purpose. Indeed, various modifications
- 20 of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.

- It is further to be understood that all base sizes or amino acid sizes, and all molecular
- 25 weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description.

Various publications, patent applications and patents are cited herein, the disclosures of which are incorporated by reference in their entireties.

WHAT IS CLAIMED IS:

1. A method of identifying a stem cell comprising:
 - (a) obtaining a cell sample which contains a stem cell; and
 - 5 (b) detecting the expression of BCRP by a cell in the cell sample; wherein a cell that expresses BCRP is identified as a stem cell.
2. The method of Claim 1 wherein said detecting is performed with an antibody that binds to BCRP; and wherein a stem cell is identified if it binds to said antibody.
- 10 3. The method of Claim 1 wherein said detecting is performed with a PCR probe for the nucleic acid that expresses BCRP.
4. The method of Claim 1 wherein said detecting is performed by measuring the pumping activity of the cell; wherein a stem cell is identified if it can remove Hoechst
15 33342 dye, but not Rhodamine 123 dye.
5. The method of Claim 1 further comprising:
 - (c) detecting the expression of a cell marker associated with stem cells;
 - 20 wherein a cell that expresses BCRP and expresses the cell marker is identified as a stem cell.
6. The method of Claim 5 wherein said cell marker is selected from the group consisting of EM10, CD34, Thy-1, P-gp, Ac133, and c-kit.
- 25 7. The method of Claim 5 wherein said cell marker is CD34 and wherein the cell is also CD38⁺.
8. The method of Claim 1 further comprising
 - 30 (c) detecting the expression of a lineage specific marker; wherein a cell that expresses BCRP but does not express the lineage specific marker is identified as a stem cell.

9. The method of Claim 8 wherein said stem cell is a hematopoietic stem cell and the lineage specific marker is selected from the group consisting of CD2, CD3, CD4, CD8, CD14, CD15, CD16, CD19, CD20, CD33, CD38, CD56, CD71, HLA-DR, and glycophorin A.

5

10. A method of isolating a cell that expresses BCRP comprising:
(a) obtaining a cell sample which contains a cell that expresses BCRP;
(b) detecting the expression of BCRP by a cell in the cell sample; and
(c) isolating the cell that expresses BCRP.

10

11. A method of isolating a cell that expresses BCRP comprising:
(a) obtaining a cell sample which contains a cell that expresses BCRP;
(b) contacting the cell sample with an antibody that binds to BCRP; and
(c) isolating a cell from the cell sample that binds to said antibody;

15 wherein the isolated cell is a cell that expresses BCRP.

12. The method of Claim 11, wherein said isolating is performed by flow cytometry.

20 13. The method of Claim 12, wherein said antibody has a fluorescent label and said isolating is performed by fluorescence-activated cell sorting.

14. An isolated stem cell obtained by the method of Claim 11.

25 15. A cell population enriched for stem cells obtained by a method comprising:
(a) obtaining a mixed population of cells which comprises both stem cells and cells that are not stem cells; and
(b) separating the cells of the mixed population of cells into two individual groups of cells based on whether they do or do not express BCRP; wherein the group
30 of cells that express BCRP is a cell population enriched for stem cells.

16. An antibody that recognizes an extracellular portion of a BCRP; wherein said extracellular portion of the BCRP is in its natural conformation.
17. The antibody of Claim 16 wherein the BCRP is on the surface of a viable stem
5 cell.
18. A method for determining the amount of BCRP expressed in a human subject having or suspected of having leukemia comprising:
- 10 (a) obtaining a leukemic cell from the subject; and
(b) determining the amount of BCRP expressed in the leukemic cell.
19. The method of Claim 18, wherein the leukemia is acute myelogenous leukemia (AML).
- 15 20. The method of claim 19 wherein the leukemic cell is a blast cell.

1340-1-021 CIP2 (Sheet 1 of 1)

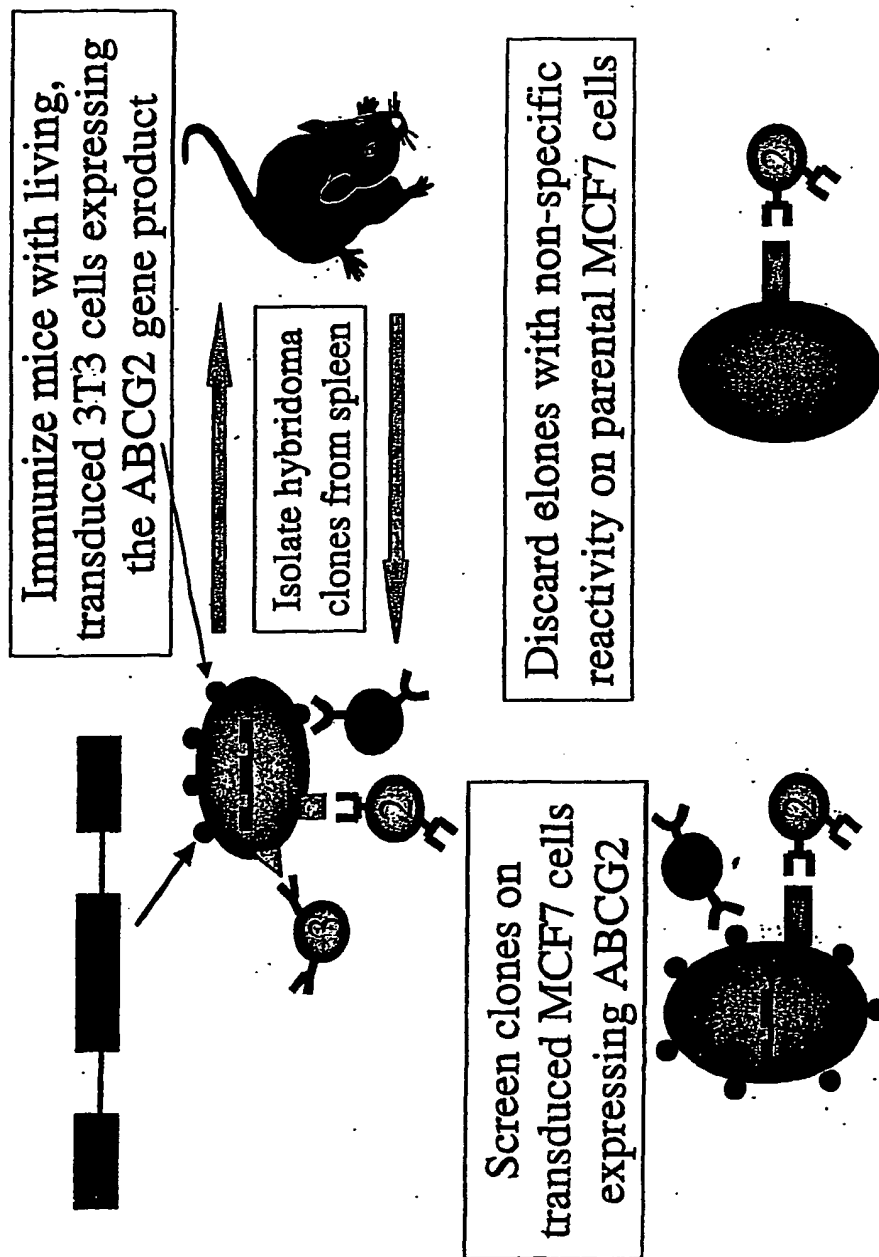


Figure 1

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Pro Thr Gly Gly Gly Lys Ser Ser Leu Leu Asp Val Leu Ala Ala Arg
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Lys Asp Pro Ser Gly Leu Ser Gly Asp Val Leu Ile Asn Gly Ala Pro
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Arg Pro Ala Asn Phe Lys Cys Asn Ser Gly Tyr Val Val Gln Asp Asp
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Val Val Met Gly Thr Leu Thr Val Arg Glu Asn Leu Gln Phe Ser Ala
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Ala Leu Arg Leu Ala Thr Thr Met Thr Asn His Glu Lys Asn Glu Arg
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Ile Asn Arg Val Ile Glu Glu Leu Gly Leu Asp Lys Val Ala Asp Ser
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Lys Val Gly Thr Gln Phe Ile Arg Gly Val Ser Gly Gly Glu Arg Lys
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Arg Thr Ser Ile Gly Met Glu Leu Ile Thr Asp Pro Ser Ile Leu Ser
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Leu Asp Glu Pro Thr Thr Gly Leu Asp Ser Ser Thr Ala Asn Ala Val
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Leu Leu Leu Leu Lys Arg Met Ser Lys Gln Gly Arg Thr Ile Ile Phe
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Ser Ile His Gln Pro Arg Tyr Ser Ile Phe Lys Leu Phe Asp Ser Leu
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 Val Ala Leu Asn Arg Glu Glu Asp Phe Lys Ala Thr Glu Ile Ile Glu
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 Pro Ser Lys Gln Asp Lys Pro Leu Ile Glu Lys Leu Ala Glu Ile Tyr
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 Val Asn Ser Ser Phe Tyr Lys Glu Thr Lys Ala Glu Leu His Gln Leu
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 Ser Gly Gly Glu Lys Lys Lys Lys Ile Thr Val Phe Lys Glu Ile Ser
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 Tyr Thr Thr Ser Phe Cys His Gln Leu Arg Trp Val Ser Lys Arg Ser
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 Phe Lys Asn Leu Leu Gly Asn Pro Gln Ala Ser Ile Ala Gln Ile Ile
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 Val Thr Val Val Leu Gly Leu Val Ile Gly Ala Ile Tyr Phe Gly Leu
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 Lys Asn Asp Ser Thr Gly Ile Gln Asn Arg Ala Gly Val Leu Phe Phe
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 Pro Gly Leu Asn Ala Thr Gly Asn Asn Pro Cys Asn Tyr Ala Thr Cys
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Thr Gly Glu Glu Tyr Leu Val Lys Gln Gly Ile Asp Leu Ser Pro Trp
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Arg Thr Ile Gly Pro Trp Leu Ser Trp Leu Gln Tyr Phe Ser Ile Pro
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Arg Tyr Gly Phe Thr Ala Leu Gln Tyr Asn Glu Phe Leu Gly Gln Glu
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Phe Cys Pro Gly Phe Asn Val Thr Asp Asn Ser Thr Cys Val Asn Ser
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Tyr Ala Ile Cys Thr Gly Asn Glu Tyr Leu Ile Asn Gln Gly Ile Glu
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Leu Ser Pro Trp Gly Leu Trp Lys Asn His Val Ala Leu Ala Cys Met
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 Lys Ser Gly Phe Leu Val Arg Lys Thr Val Glu Lys Glu Ile Leu Ser
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 Thr Gly Gly Gly Lys Ser Ser Leu Leu Asp Val Leu Ala Ala Arg Lys
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 Asp Pro Lys Gly Leu Ser Gly Asp Val Leu Ile Asn Gly Ala Pro Gln
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 Pro Ala His Phe Lys Cys Cys Ser Gly Tyr Val Val Gln Asp Asp Val
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 Asp Glu Pro Thr Thr Gly Leu Asp Ser Ser Thr Ala Asn Ala Val Leu
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 Leu Glu Tyr Phe Ala Ser Ala Gly Tyr His Cys Glu Pro Tyr Asn Asn
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 Pro Ala Asp Phe Phe Leu Asp Val Ile Asn Gly Asp Ser Ser Ala Val
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 Phe Lys Asn Leu Leu Gly Asn Pro Gln Ala Ser Val Ala Gln Leu Ile
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 Lys Tyr Asp Ala Ala Gly Met Gln Asn Arg Ala Gly Val Leu Phe Phe
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 Pro Gly Phe Asn Val Thr Asp Asn Ser Thr Cys Val Asn Ser Tyr Ala
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 Pro Trp Gly Leu Trp Lys Asn His Val Ala Leu Ala Cys Met Ile Ile
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Lys Ser Gly Phe Leu Pro Cys Arg Lys Pro Val Glu Lys Glu Ile Leu
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Pro Thr Gly Gly Gly Lys Ser Ser Leu Leu Asp Val Leu Ala Ala Arg
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Ile Asn Arg Val Ile Gln Glu Leu Gly Leu Asp Lys Val Ala Asp Ser
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Lys Val Gly Thr Gln Phe Ile Arg Gly Val Ser Gly Gly Glu Arg Lys
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Ala Leu Gly Tyr Phe Glu Ser Ala Gly Tyr His Cys Glu Ala Tyr Asn
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Asn Pro Ala Asp Phe Phe Leu Asp Ile Ile Asn Gly Asp Ser Thr Ala
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